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IGF-II/Man-6-P receptors in rat kidney: Apical localization in proximal tubule cells

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IGF-II/Man-6-P receptors in rat kidney: Apical localization in proximal tubule cells. The present study was performed to determine the localization of insulin-like growth factor II (IGF-II) binding in the rat nephron and, furthermore, to determine directly the distribution of the IGF-II/Man-6-P receptor in the proximal tubule. The IGF-II/Man-6-P receptor was visualized by immunohisto- and cytochemical techniques, and the binding of IGF-II was shown by light and electron microscope autoradiography applying ^{125}I-IGF-II directly on cryosections. The tubular uptake of IGF-II was studied by autoradiography after *in vivo* injection of ^{125}I-IGF-II. The light microscope immunolabeling was confined to a relatively narrow band in the apical part of the proximal tubule cells just below the brush border, and was strongest in the first portion of the proximal tubule, segment 1 (S1), with decreasing intensity in segment 2 (S2) and weaker in segment 3 (S3). Several cytoplasmic bodies were labeled but no label was observed in the basolateral part of the cells. We did not detect any labeling of glomeruli or other segments of the nephron. Electron microscope immunocytochemistry in the proximal tubule revealed a strong labeling in apical endocytic invaginations, small endocytic vacuoles, and large endocytic vacuoles, weaker in lysosomes and dense apical tubules. Light microscope autoradiography after incubation of cryosections with labeled IGF-II demonstrated an intense labeling just beneath the brush border, especially in S1 of the proximal tubule, and, in addition, the corresponding electron microscope autoradiography revealed an intense labeling over large endocytic vacuoles and lysosomes. These results were confirmed and extended by competitive binding experiments and quantitative immunocytochemistry. After i.v. injection of ^{125}I-IGF-II autoradiographic grains were almost exclusively observed in the electronmicroscope over endocytic vacuoles and lysosomes in the S1 of the proximal tubule. The present study shows that the IGF-II/Man-6-P receptor is located in components of the vacuolar system in the apical cytoplasm of rat renal proximal convoluted tubule cells, mainly in S1. This localization was seen to be consistent using various immunolabeling and autoradiographic techniques *in vivo* and *in vitro*.

Insulin-like growth factor II (IGF-II) is a polypeptide (molecular wt 7,500) with partial structural homology with both insulin and insulin-like growth factor I (IGF-I) [1]. IGF-II is produced in a number of tissues and the production is probably less GH-dependent than IGF-I production [2]. It has also been suggested that IGFs produced in tissues may act as local growth factors by autocrine or paracrine action [3, 4].

The IGF-II/Man-6-P receptor is structurally unrelated to the IGF-I and insulin receptors, and consists of a single chain (M_r = 220 kDa) transmembrane glycoprotein without tyrosine-kinase activity [5, 6]. Morgan et al [6] demonstrated that the amino acid sequence of the human IGF-II receptor is 80% homologous to the bovine cationic mannose-6-phosphate receptor, and later biochemical studies have supported this homology [7]. The IGFs bind to cell surface receptors on a variety of target cells and cause both metabolic effects and changes in the rate of protein synthesis and growth [8]. IGF-I also binds to the IGF-II/Man-6-P receptor, but with much lower affinity than IGF-II, while the IGF-II/Man-6-P receptor does not bind insulin at all [5].

Previous studies have described the existence of IGF-II/Man-6-P receptors in the plasma membrane of a variety of different cell types, including renal glomeruli and tubules [9, 10]; furthermore, mRNA expression for IGF-II/Man-6-P receptors has been shown in kidney tissue [11]. In the renal proximal tubule the IGF-II/Man-6-P receptor has been characterized both in the brush border and basolateral membrane vesicle fractions [10], and the relative distribution was reported to be symmetrical [10]. A physiological effect from activated IGF-II/Man-6-P receptors in the luminal membranes in the proximal tubule has not been demonstrated. Effects such as phospholipase C activation and stimulation of Na^+-H^+ exchange in proximal tubules have been ascribed to action from basolateral receptors [12, 13]. Receptors from renal basolateral membranes have been shown to be activated by mannose-6-phosphate containing peptides in addition to activation by IGF-II [14].

The aim of the present study was to describe the distribution of the IGF-II/Man-6-P receptor in the rat kidney, both with respect to localization in the different segments of the nephron and to the exact cellular localization. In this study a very consistent localization of the IGF-II/Man-6-P receptor in rat kidney was observed using several different techniques: immunohisto- and cytochemistry for localization of the receptor and autoradiography at the light and electron microscope levels *in vivo* and *in vitro* for localization of IGF-II binding. We report here that the IGF-II/Man-6-P receptor is localized in the apical

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part and in cytoplasmic bodies in rat kidney proximal tubule cells.

Methods

Animals

Male Wistar rats (Møllegaard Avlslab., Eiby, Denmark), weighing between 240 and 300 grams, were used for all experiments.

Antibody

In the present study a specific rabbit anti-rat IGF-II/Man-6-P receptor antiserum 3637 [15] was used for immunohistochemistry and immunoblotting. This antiserum does not cross react with the IGF-I or insulin receptors, nor does it recognize IGF binding proteins or the cation-dependent Man-6-P receptor [7, 15]. For preabsorption control experiments, rat IGF-II/Man-6-P receptor was purified from livers of 150 g Sprague-Dawley rats. Livers were homogenized and a plasma membrane preparation was obtained by differential ultracentrifugation. The microsomal membrane fraction was solubilized in 1% Triton-X 100. The solubilized plasma membranes were incubated with a beta-galactosidase-Sepharose affinity column, and IGF-II/M-6-P receptors were eluted from the column after one hour of incubation of the column with 10 mM M-6-P [7]. Protein content in the receptor preparation was measured using Pierce BCA assay (Pierce Chemical Co, Rockford, Illinois, USA; Catalog no. 23225).

Immunoblotting

Renal cortical tissue from rats, 100 mg aliquots were homogenized, solubilized in 1 ml 1% Triton X-100 in 20 mM Tris, 150 mM NaCl and 1 mM phenylmethanosulfonyl fluoride and centrifuged for two minutes in an Eppendorf centrifuge. From the supernatant 20 μl was applied to a sodium dodecyl sulfonate gel with a 4 to 16% polyacrylamide gradient, electroblotted for 1.5 hours at 0.2 amps onto an Immobilon filter (Millipore). The filter was blocked for two hours in 2% nonfat dry milk, incubated for two hours with the IGF-II/Man-6-P antibody diluted 1:1000 in 0.2% nonfat dry milk, washed 3 × 15 minutes in PBS with 0.05% Tween-20, incubated with alkaline phosphatase conjugated anti-rabbit antibody (Dako A/S, Glostrup, Denmark) in a 1:2000 dilution in 0.2% nonfat dry milk, and finally washed. Staining was visualized using 5-bromo-4-chloro-3-indolyl-phosphate (0.125 mg/ml) and Nitro Blue Tetrazolium (0.25 mg/ml) as substrate.

Tracers and peptides

[3-(^{125}I)iodotyrosyl] IGF-II with a specific activity of 2,000 Ci/mmol (purchased from Amersham International, Amersham, Bucks, UK) or human ^{125}I-IGF-II (400 Ci/mmol) labeled by the chloramine-T method was used in all autoradiographic experiments. Unlabeled biosynthetical IGF-I and IGF-II were purchased from Bachem (Bubendorf, Switzerland), while unlabeled porcine insulin was provided by Novo Nordisk A/S (Bagsværd, Denmark). Cytochrome C and lysozyme were purchased from Sigma Chemical Co.

Immunohistochemistry and immunocytochemistry

The rats for immunohisto- or immunocytochemistry studies were anaesthetized i.p. with sodium pentobarbital (65 mg/kg body wt), and the kidneys were fixed by retrograde perfusion through the abdominal aorta with 8% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.2. Then tissue blocks from cortex, outer and inner medulla were further fixed by immersion in the same fixative for one hour, immersed for 30 minutes in 2.3 M sucrose containing 2% paraformaldehyde, and finally frozen in liquid nitrogen.

Sections were obtained by cryoultramicrotomy using a Reichert-Jung FC 4D cryoultramicrotome at 160 to 180 K. For light microscopy 0.80 μm sections were placed on gelatin-coated glass slides. For electron microscopy 85 nm sections were placed on 300 mesh Ni-grids. All sections were preincubated in PBS containing 0.1% to 0.3% nonfat dry milk and 0.05 M glycine, except the sections for immunofluorescence which were preincubated in PBS containing 0.1% gelatin, 0.5% BSA and 0.02 M glycine.

For immunolabeling, the sections were incubated with the primary antibody (IGF-II/Man-6-P-receptor antibody) at 4°C overnight at different dilutions (1:50 to 1:150) in the same buffer as described above but without glycine, and then washed in the same buffer. For light microscopy, the sections were incubated either with FITC-conjugated swine anti-rabbit immunoglobulins for immunofluorescence (Dako) or with peroxidase-conjugated affinity-isolated goat anti-rabbit immunoglobulins for immunohistochemistry (Dako). The peroxidase was visualized with diaminobenzidine (10 min incubation) and the sections were washed. For electron microscopy, the sections were incubated for two hours at 4°C with 5 nm goat anti-rabbit gold. The sections for electron microscopy were finally covered with 2% methylcellulose containing 0.3% uranyl acetate as described by Tokuyasu [16] and modified by Griffiths et al [17].

For quantitative analysis of immunocytochemical localization of IGF-II/Man-6-P receptors, proximal tubules were photographed at random at a primary magnification of ×20,000 and enlarged to a final magnification of ×60,000. Gold particles were counted over the different organelles in the cells, and the areas of the same regions were determined by point counting [18] using a lattice square test system with a distance between lines of 25 mm corresponding to 0.42 μm. The values were expressed as a percentage of the total area of the cells analyzed.

For immunolabeling, the following controls were performed: (1.) incubation with a nonspecific rabbit serum (Dako); (2.) incubation with the specific antibody, diluted 1:150, preabsorbed with purified receptor (see above), 6 mg/ml; (3.) without primary antibody; and (4.) without primary and secondary antibodies.

In vitro *incubation of sections with ^{125}I-IGF-II*

For autoradiography, sections were incubated with ^{125}I-labeled IGF-II (about 1 μCi/ml) either alone or together with different proteins for competitive binding studies (unlabeled IGF-II, IGF-I, insulin, cytochrome C and lysozyme) at varying concentrations diluted in PBS buffer containing 0.1% nonfat dry milk. The sections were washed and subsequently fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffers, pH 7.2, for

10 minutes and then washed. For light microscope autoradiography the sections were dipped in LM-1 emulsion (Amersham), exposed for one to two weeks, developed in Kodak D19 developer, fixed in sodium thiosulphate, and subsequently stained with toluidine blue. For electron microscope autoradiography the sections were covered with 2% methylcellulose containing 0.3% uranyl acetate as described above and then coated with carbon. Autoradiographic emulsion (Ilford L4) was applied by a modified wire loop method [19], exposed for one to two weeks, developed in Kodak D19, and fixed in sodium thiosulphate.

Preparation of renal tissue after i.v. injection of ^{125}I-IGF-II

Rats anaesthetized i.p. with sodium pentobarbital (65 mg/kg of body wt) were injected in the femoral vein with 1 ml of ^{125}I-labeled IGF-II (40 μCi) with a specific activity of 400 Ci/mmol. Five minutes after injection the kidneys were fixed by perfusion as above with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, further fixed by immersion in the same fixative for a minimum of 12 hours, and postfixed in 1% OsO_4 for 60 minutes. Subsequently the tissue was dehydrated in graded alcohols (70, 90, 96, and 99%) and embedded in Epon 812 in flat molds.

Thin sections were cut on an LKB Ultrotome III, mounted on 100 mesh copper grids and stained with lead citrate and uranyl acetate. Ilford L4 emulsion was applied to the grids as described above, developed in Kodak D 19 (90 seconds, 20°C) and fixed in a 20% sodium thiosulphate solution for 120 seconds.

Microscopy

The FITC-labeled sections were studied in a Zeiss Axiophot microscope, the peroxidase labeled sections in a Leitz orthoplane microscope, and the electron microscope sections in a JEOL 100 CX electron microscope.

Results

Immunoblotting

The immunoblots demonstrated labeling of only one band with an apparent molecular weight of 220 to 250 kDa corresponding to the IGF-II/Man-6-P receptor (Fig. 1), as previously shown [7, 9, 15].

Immunohistochemistry

Immunolabeling for the IGF-II/Man-6-P receptor was observed only in the presence of the specific antibody and was confined to a narrow band in the apical part of the proximal tubule cells just below the brush border as revealed both by immunoperoxidase (Fig. 2) and immunofluorescence (Fig. 3 a and b). In addition, cytoplasmic bodies were labeled in the proximal tubule, especially in segments 1 and 2 (Figs. 2 and 3). The labeling in segment 3 was weaker than that in segments 1 and 2 (Fig. 3 a and b). It should be emphasized that no labeling was observed in the brush border or the basolateral parts of the proximal tubule cells. Furthermore, we observed no labeling in the glomeruli, in other segments of the nephron, or in the collecting duct system (Figs. 2 and 3).

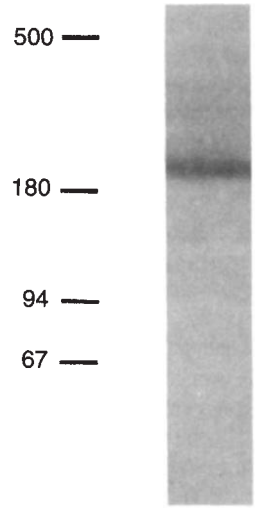

Fig. 1. *Immunoblot of homogenate from rat renal cortex using the anti-IGF-II receptor serum.* The stained protein band had a molecular weight of about 220,000 to 250,000 under non-reducing conditions corresponding to the IGF-II/Man-6-P receptor. Molecular weight markers are α_2-macroglobulin receptor (nonreduced 500 kDa), α_2-macroglobulin (reduced 180 kDa), phosphorylase b (reduced 94 kDa) and albumin (reduced 67 kDa).

Light microscope autoradiography

Cryosections incubated with ^{125}I-labeled IGF-II revealed a significant labeling of cross sections of proximal tubules. Cryosections obtained from the juxtamedullary part of cortex demonstrated an intense labeling in segment 1 (S1) with decreasing intensity in segment 2 (S2) and weaker in segment 3 (S3) (Fig. 4 a and b). The label was confined mainly to the apical part of the proximal tubule cells just below the brush border and there was no labeling of the brush border. In addition to the apical labeling of especially segment 1 (Fig. 5a) there was a certain cytoplasmic labeling. No other parts of the nephron or the glomeruli showed any specific labeling.

In competitive binding experiments the apical binding of ^{125}I-IGF-II was totally inhibited by unlabeled IGF-II at a concentration of 10^{-6} M, while at the same concentration of unlabeled IGF-I there was still a little labeling left (Figs. 5 b–d). At a concentration of 10^{-5} M of unlabeled IGF-I there was no reaction left and thus IGF-I was more than 10 times less effective than IGF-II in blocking the ^{125}I-IGF-II binding. Insulin at concentrations up to 10^{-5} M did not inhibit binding of ^{125}I-IGF-II, nor did cytochrome C or lysozyme at similar concentrations.

Immunocytochemistry

In general the immunogold-labeling of the proximal tubules was most intense over S1, decreasing in S2, and relatively weaker in S3. In all segments of the proximal tubules, the apical endocytic invaginations, small and large endocytic vacuoles were labeled with gold particles (Fig. 6 a and b). Lysosome-like bodies, dense apical tubules and brush border showed less labeling and almost no labeling was observed over nuclei and mitochondria. No significant labeling was observed in other parts of the nephron.

A semiquantitation of the distribution of IGF-II/Man-6-P

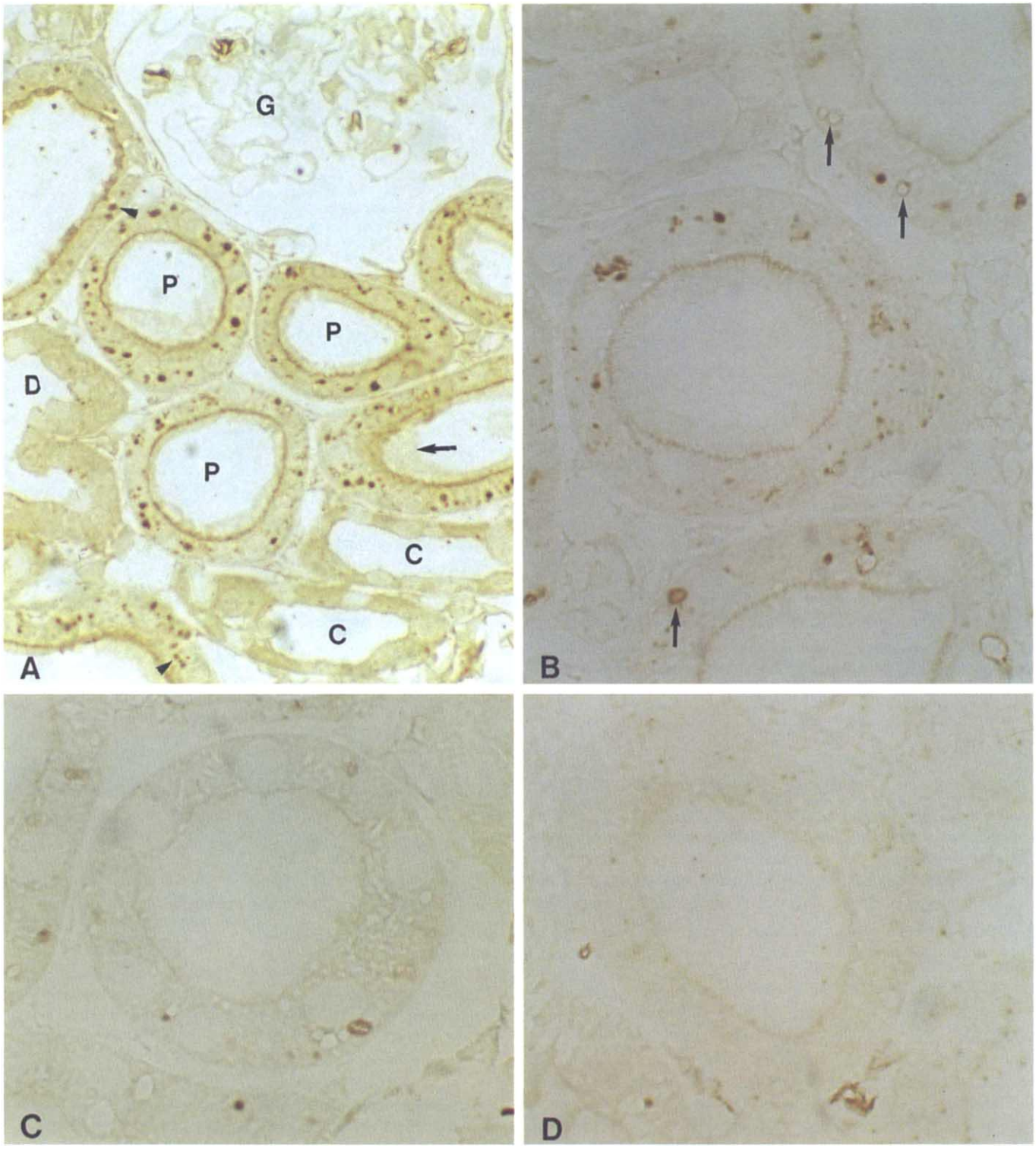

Fig. 2. *Immunoperoxidase staining (antiserum 3637, 1:100) for IGF-II/Man-6-P receptors is seen as a narrow band in the apical part of the proximal tubules (P) just below the brush border (arrow).* Cytoplasmic bodies (arrowheads) in proximal tubules are also labeled. No labeling is seen in the basal part of the proximal tubule cells corresponding to the basolateral membranes. Furthermore, glomeruli (G), distal tubules (D), and collecting ducts (C) are not labeled. ×550. **B.** Immunoperoxidase staining (antiserum 3637, 1:150) of proximal tubules at higher magnification. The staining is again seen as a narrow bond just below the brush border. The staining of cytoplasmic bodies often appears as ring-like structures (arrows). ×1,200. **C.** Absorption control (antiserum 3637, 1:150, preabsorbed with purified receptor, 6 mg/ml) of parallel section to B. Only a weak staining is seen below the brush border and the staining of the cytoplasmic bodies has almost disappeared. ×1,200. **D.** Incubation with non-immune serum 1:150. Parallel section to B and C. A very weak staining is seen below the brush border and no cytoplasmic staining is seen. ×1,200.

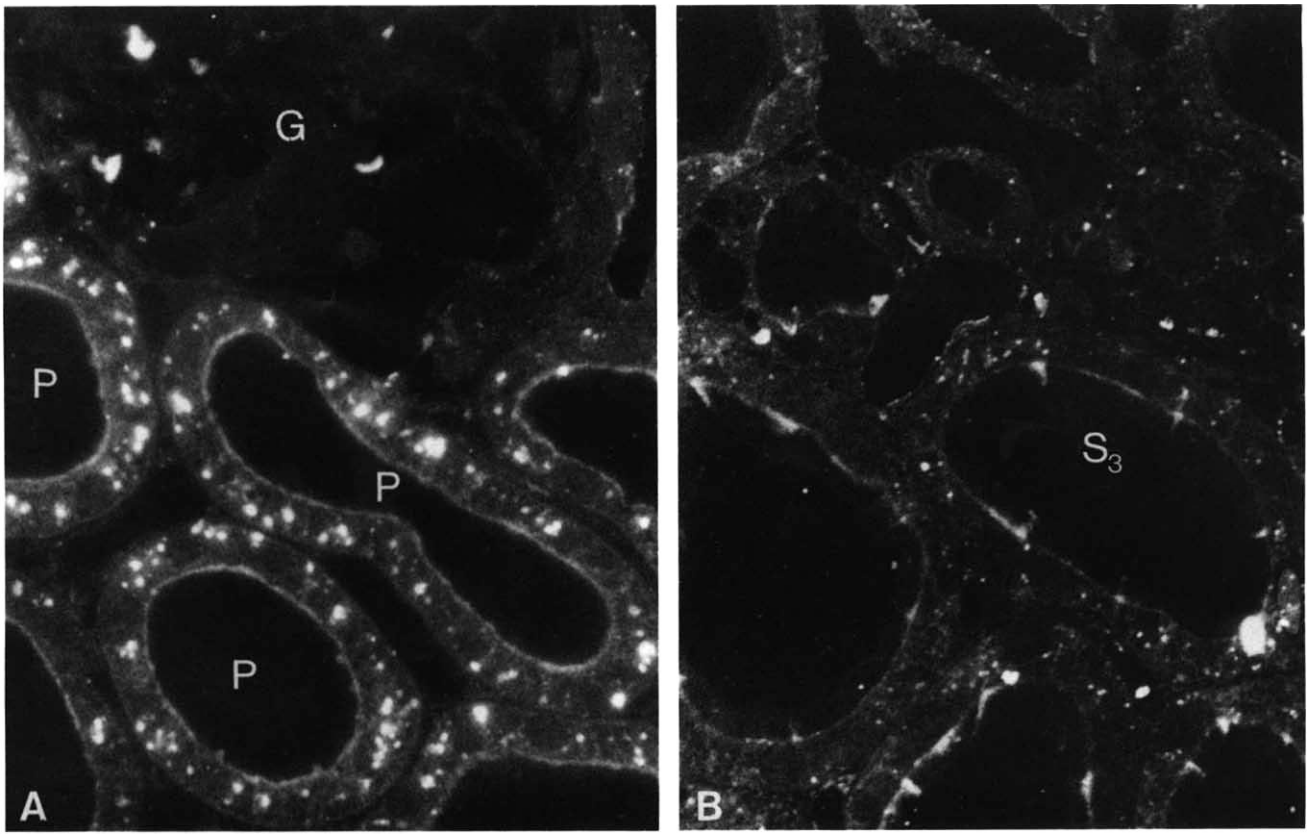

Fig. 3. *Immunofluorescence micrographs (antiserum 3637, 1:100) showing a narrow band corresponding to IGF-II/Man-6-P receptors in the apical part of proximal tubules.* No labeling of the basolateral membranes is seen. **A.** Micrograph from cortex demonstrating labeling of cytoplasmic bodies besides the apical labeling of the renal proximal convoluted (P) tubules, segments 1 and 2. No labeling is seen in glomeruli (G). ×490. **B.** Micrograph from outer medulla. The labeling found in the proximal tubules (segment 3, S3) is weaker than that seen in segment 1 and segment 2 in A. Also the labeling of cytoplasmic bodies is more sparse. ×640.

receptors in the proximal tubule is shown in Table 1. The highest density of gold particles was seen in the apical part of the cells, over endocytic invaginations, small endocytic vacuoles and large endocytic vacuoles. Also an accumulation was observed in lysosomes and over the brush border, whereas the relative density over the mitochondria, nuclei and cytoplasm was less than 1.

Electron microscope autoradiography in vitro

Large endocytic vacuoles which had an electron-lucent interior were occasionally heavily labeled, and so was the apical part of the cytoplasm probably corresponding to endocytic invaginations and small endocytic vacuoles (Fig. 7 a and b). In many cells of the proximal tubule the lysosome-like bodies identified by their electron dense appearance were heavily labeled with grains (Fig. 7c). However, there was also a low apparent unspecific background labeling over the cytoplasm.

Electron microscope autoradiography after i.v. injection of ^{125}I-IGF-II

Autoradiographic grains were found exclusively over the proximal tubules. The vast majority of the grains appeared in the first segment of the proximal tubule. The labeling was much less pronounced in the second and third segments of the proximal tubule, S2 and S3. The grains in all segments of the proximal tubule were mainly located over small and large endocytic vacuoles, dense apical tubules, and lysosomes (Fig. 8). There was no labeling over any other part of the cells.

Discussion

In the present study we determined the localization of IGF-II/Man-6-P receptors in rat renal proximal tubules by means of immunohistochemical, immunocytochemical, and autoradiographic techniques *in vivo* and *in vitro*. There was a high degree of consistency between the different methods regarding localization of the IGF-II/Man-6-P receptors in the nephron both at cellular and subcellular levels. The binding sites were confined to components of the vacuolar system related to endocytosis in the apical part of proximal tubule cells. Furthermore, the labeling intensity of both the immunolocalization of the receptor and the specific binding of IGF-II was highest in segment 1, lower in segment 2 and lowest in segment 3. There was no detectable labeling of basolateral membranes in proximal tubules and no labeling in other parts of the nephron was observed.

The IGF-II/Man-6-P receptor binds IGF-II with high affinity, IGF-I with a lower affinity and does not bind insulin at all [5]. In our competitive binding experiments the ^{125}I-IGF-II binding was almost completely inhibited by unlabeled IGF-II. The binding was also to a high extent inhibited by unlabeled IGF-I,

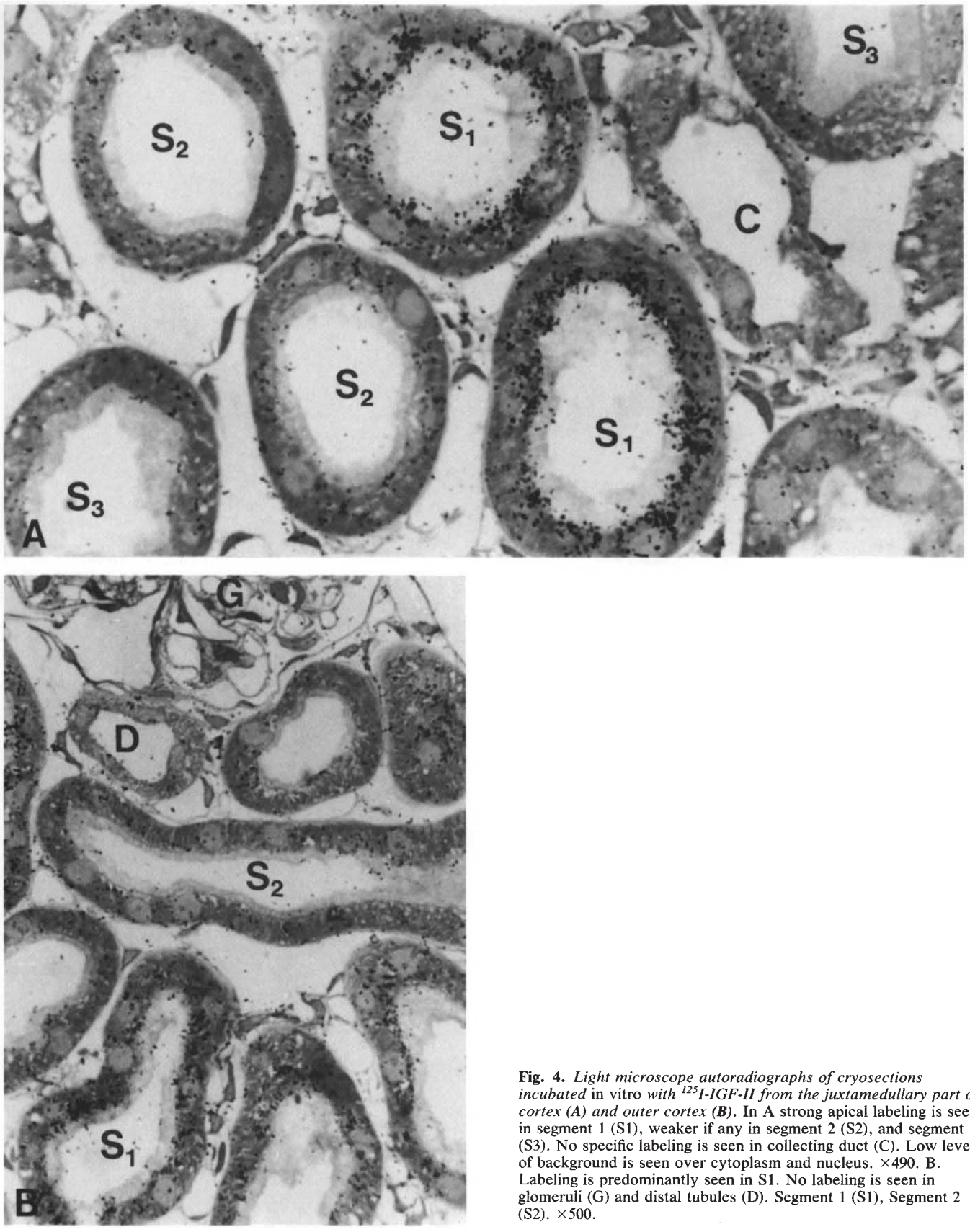

Fig. 4. *Light microscope autoradiographs of cryosections incubated* in vitro *with* ^{125}*I-IGF-II from the juxtamedullary part of cortex (A) and outer cortex (B).* In A strong apical labeling is seen in segment 1 (S1), weaker if any in segment 2 (S2), and segment 3 (S3). No specific labeling is seen in collecting duct (C). Low level of background is seen over cytoplasm and nucleus. ×490. B. Labeling is predominantly seen in S1. No labeling is seen in glomeruli (G) and distal tubules (D). Segment 1 (S1), Segment 2 (S2). ×500.

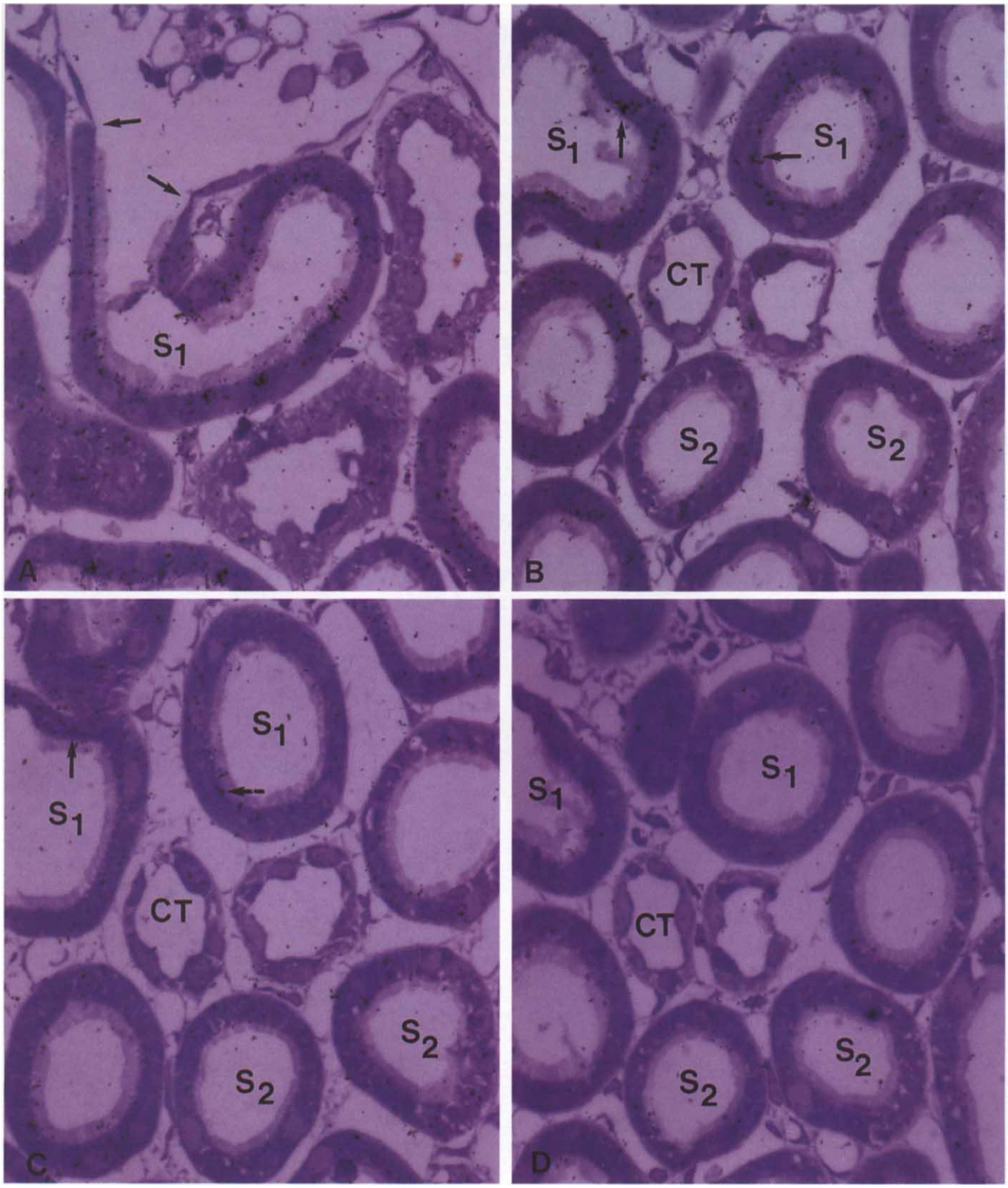

Fig. 5. *Light microscope autoradiography of cryosections from outer cortex incubated* in vitro *with* ^{125}*I-IGF-II.* Observe that B, C and D are from neighboring sections. During incubation 10^{-6} M cold IGF-I was added to C, and 10^{-6} M cold IGF-II to D. **A.** Labeling is seen very heavily concentrated in the initial part of the proximal tubule (S1), here seen connected to the Bowman's capsule (arrows). ×750. **B.** Heavy labeling is seen in segment 1 (S1), especially just below the brush border (arrows); only weak labeling is seen in S2. No specific labeling is observed in connecting tubules (CT). ×750. **C.** The labeling has extensively decreased in S1, although, a little labeling is still seen (arrows). ×750. **D.** The labeling has almost disappeared. ×750.

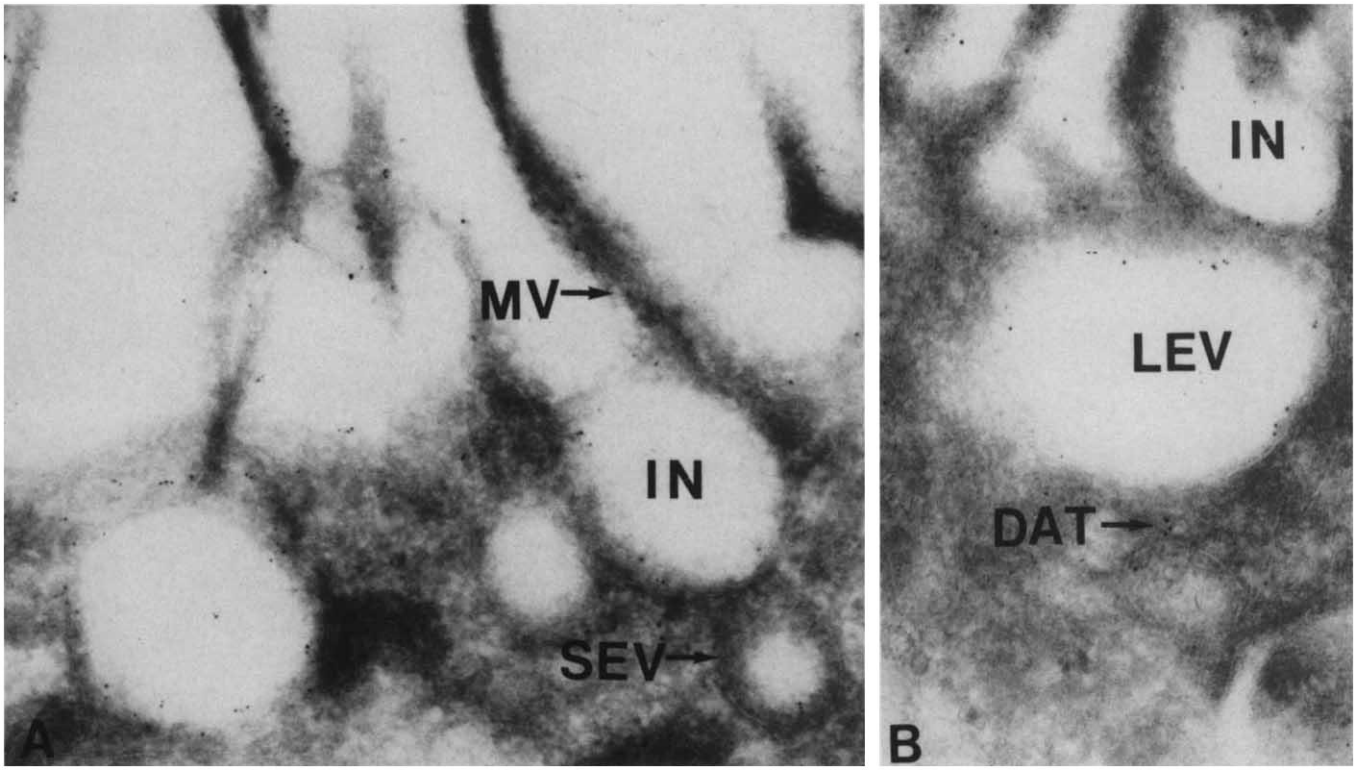

Fig. 6. *Ultrathin cryosections of the renal proximal tubules immunolabeled for IGF-II/Man-6-P receptor using protein A-gold (5 nm).* **A.** Gold particles are found in the apical part of the renal proximal tubule cells mainly in invaginations (IN) and small endocytic vacuoles (SEV). Microvilli (MV). ×60,000. **B.** Labeling is seen in large endocytic vacuole (LEV), invaginations (IN) and dense apical tubules (DAT). ×75,000.

Table 1. Distribution of IGF-II/Man-6-P receptors in rat renal proximal tubule[a]

	CYT	MIT	NUC	LEV	SEV	IN	LY	DAT	BB	Total
Gold particles, *no.*	444	537	529	451	406	751	83	146	739	4086
Gold particles, %	10.9	13.1	12.9	11.0	10.0	18.4	2.0	3.6	18.1	
% Area	41.5	13.9	22.6	3.6	1.9	3.8	0.9	3.0	8.7	
Relative density[b] *% gold/% area*	0.26	0.94	0.57	3.1	5.3	4.8	2.2	1.2	2.1	

Abbreviations are: CYT, cytoplasm; MIT, mitochondria; NUC, nucleus; LEV, large endocytic vacuoles; SEV, small endocytic vacuoles; LY, lysosomes; DAT, dense apical tubules; BB, brush border.

[a] Total cell area examined constituted 1003 μm^2.

[b] Relative density was calculated as percentage gold particles divided by percentage area of the different cell organelles.

but not inhibited by insulin, cytochrome C or lysozyme. Thus the competition experiments further substantiated that the receptors, which were demonstrated in this study to be distributed over the apical part of the proximal tubule cells, were authentic type II IGF receptors rather than other types of receptors (that is, insulin or type I IGF receptor) or scavenger receptors for endocytic reabsorption of proteins in the proximal tubule. The limited cellular labeling seen in the light microscopic autoradiography was, at least in part, due to late endosome/pre-lysosomal labeling as revealed by the electron microscopic autoradiography (Fig. 7c).

The IGF-II/Man-6-P receptor has been localized and characterized in many tissues in various species, including humans [21]. With the increasing interest regarding the role of growth hormones including IGF-II in renal physiology and pathophysiology [reviewed in 20], a detailed knowledge of the sites of IGF-II binding in the kidney seems pertinent. However, only few studies have focused on the renal distribution of the IGF-II/Man-6-P receptor. Various localizations have been suggested for the IGF-II/Man-6-P receptors in the kidney in previous studies, such as glomeruli [9, 21] and proximal tubules [10, 21, 22]. Investigations using immunohistochemical techniques demonstrated IGF-II/Man-6-P receptors on the inner surface of renal tubules and on Bowman's capsule of the glomeruli [21].

Studies carried out using isolated basolateral and brush border membrane fractions have shown that the distribution of IGF-II/Man-6-P receptors between basolateral and brush border membranes was largely symmetrical [10] [reviewed in 20]. IGF-I receptors, in contrast, were shown to be asymmetrically distributed being predominantly basolateral [10]. In the present study using different techniques, IGF-II/Man-6-P receptors and binding sites were observed only in the apical part of the

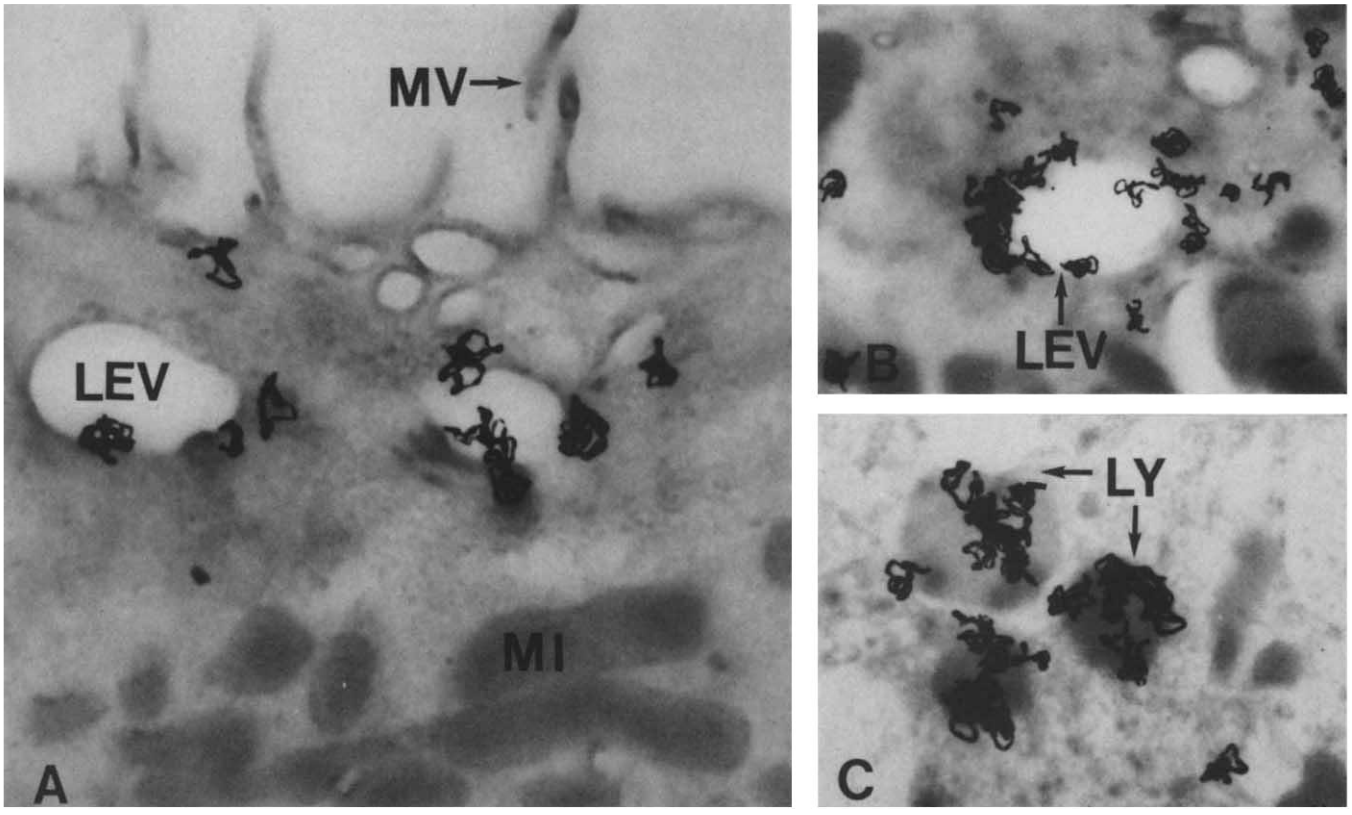

Fig. 7. *A. Electron microscope autoradiograph of proximal tubule cells from ultrathin cryosections incubated with ^{125}I-IGF-II.* Autoradiographic grains are located over the apical part of the proximal tubule cell. Large endocytic vacuoles (LEV), microvilli (MV), and mitochondria (MI). ×27,000 **B.** Demonstration of autoradiographic grains heavily concentrated over large endocytic vacuole (LEV). ×17,000 **C.** Autoradiographic grains located over lysosomes (LY). ×18,000.

proximal tubule cells. The discrepancy between the present results and the above-cited investigations regarding basolateral localization of receptors [10] is at present unknown but there may be different explanations. (1) The reason could reside in experimental procedures. It is well recognized that cell fractionation methods may suffer from limited cross-contamination of the different fractions obtained. The contamination can be expressed, such as the presence of brush border enzymes and basolateral Na^+/K^+-ATPase activity in the different fractions and appropriate corrections can be made. However, the present results strongly suggest the presence of the IGF-II/Man-6-P receptor in membranes of endocytic invaginations as well as in endocytic vesicles/vacuoles but not in the microvilli of the brush border. To our knowledge no markers specific for membrane obtained from endocytic invaginations have been described and it is possible that membrane material from this compartment may contaminate basolateral membrane vesicle fractions as well as brush border membrane vesicle fractions. As a consequence, it may be difficult to estimate the relative distribution of membrane proteins such as IGF-II/Man-6-P receptors located specifically in invaginations and intracellular vacuoles by techniques involving brush border and basolateral membrane vesicle fractions. Our results do not necessarily exclude localization of receptors in basolateral membranes, but the density is probably considerably lower compared to the luminal plasma membrane. Indeed, studies performed on isolated segments of proximal tubules suggest that IGF-II stimulated Na^+-H^+ exchange across the brush border membrane and changes in intracellular pH, and these actions were ascribed to basolateral receptor action [13]. In addition, IGF-II stimulated inositol triphosphate production by activation of phospholipase C [12] was observed only after incubation with basolateral membrane fractions and not with brush border membrane vesicles. (2) Hypothetically the luminal and basolateral receptors may be different, thus the used antibody would not necessarily recognize such a putative IGF-II/Man-6-P receptor subtype on the basolateral membranes if different from the recognized luminal counterpart. The immunoblotting (Fig. 1), however, showed only one band in renal cortical homogenates with a molecular weight similar to IGF-II/Man-6-P receptors demonstrated by other investigators in the kidney and other tissues [7, 9, 15]. The experiments with *in vitro* incubation of ^{125}I-IGF-II on cryosections clearly demonstrated displaceable apical binding and no significant basolateral binding. These binding studies and binding competition studies strongly confirm the immunolabeling results, thus, it appears unlikely that differences in receptor types can explain the discrepancy.

There was a clear dependence of the segmentation of the proximal tubule on the labeling intensity. By *in vitro* incubation of cryosections with ^{125}I-IGF-II, S1 segments were labeled in the apical part of the cells to a high extent but only very little labeling was confined to S2 and S3 segments. This pattern was

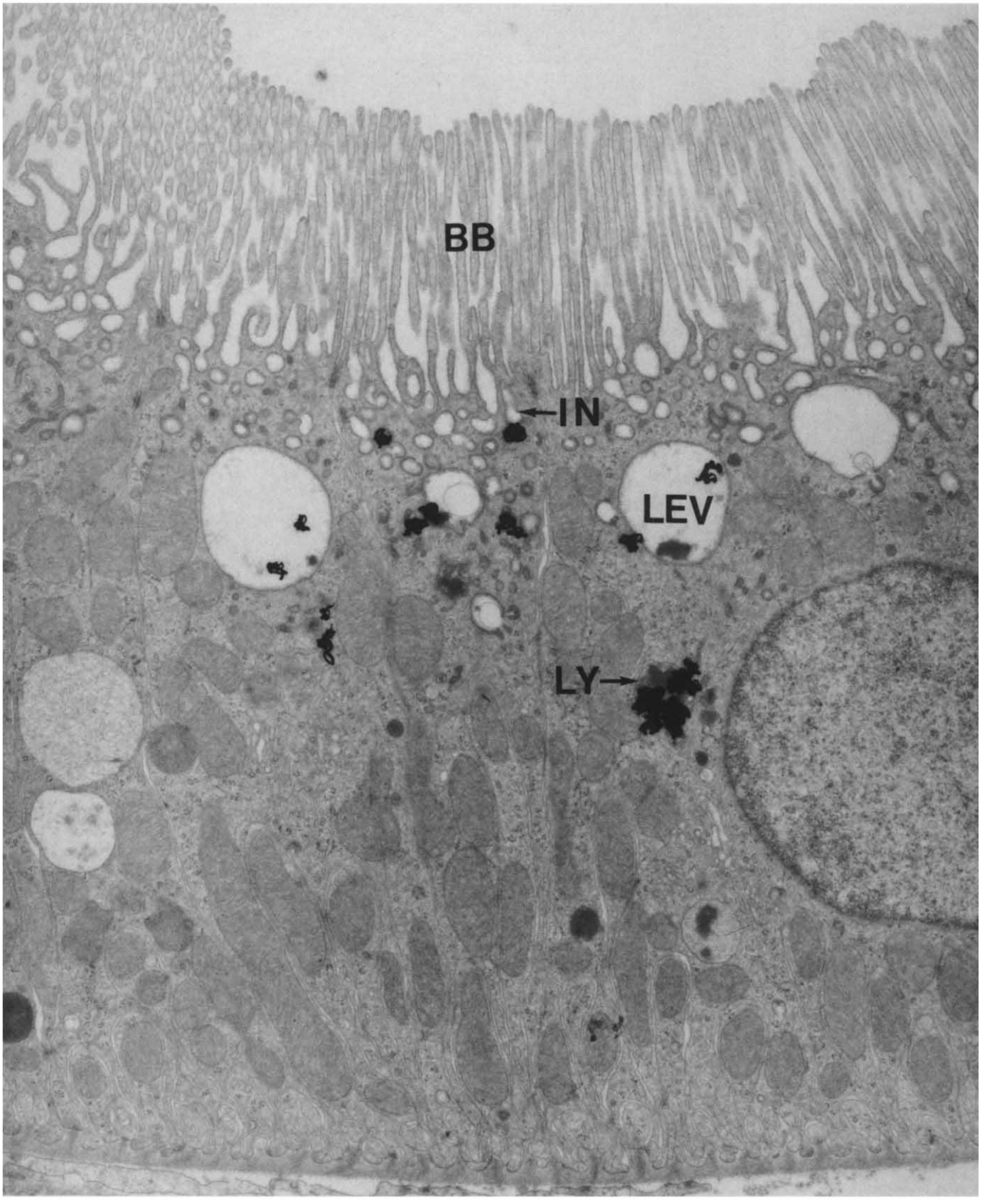

Fig. 8. *Electron microscope autoradiograph of proximal tubule S1 from rat kidney after i.v. injection of* ^{125}*I-IGF-II.* Autoradiographic grains are located over the apical part of the proximal tubule cells. Endocytic invagination (IN), large endocytic vacuoles (LEV), lysosomes (LY), brush border (BB). ×15,000.

also observed by immunocytochemistry and the labeling of S3 was considerably lower than in S1 and S2 segments. Autoradiographic grains were also almost exclusively concentrated over S1 proximal tubules after *in vivo* administration of ^{125}I-IGF-II. This may be due to the proportionally larger concentration of ^{125}I-IGF-II in the ultrafiltrate in S1 than in S2 and S3 due to progressive absorption in S1. This pattern is similar to observations in similar experiments using ^{125}I-insulin [23]. However, the endocytic uptake may also be reduced in S2 and S3 proximal tubule segments due to the lower density of receptors in the apical endocytic invaginations in segments 2 and 3 as observed in the present study. The tubular fluid in the proximal tubule S2 and S3 segments probably contain at least small amounts of IGF-II that escaped absorption in S1, since IGF-II has been determined in significant amounts in the urine [24]. After i.v. injection of ^{125}I-IGF-II, the protein was internalized by endocytosis in the proximal tubules. ^{125}I-IGF-II was concentrated in the vacuolar apparatus including the lysosomes in the apical part of proximal tubules representing receptor mediated internalization of the hormone to be degraded in the proximal tubules. Receptor mediated internalization of IGF-II has also been described in other cell types [15, 25]. The concentration of circulating IGF-II in adult rats is significantly lower than in newborn rats but is still about 40 ng/ml [26]. Thus the localization of the receptor in endocytic invaginations of proximal tubules may serve to remove IGF-II efficiently from the tubular fluid and also possibly IGF-I although with a lower efficiency due to the lower affinity of IGF-I to the IGF-II receptor. With respect to specificity of protein reabsorption in renal proximal tubule cells we have recently shown that gp330, which is located in apical endocytic invaginations and vacuoles in renal proximal tubules [27], functions as a receptor for α_2-macroglobulin receptor associated protein (α_2MRAP) [28].

The intracellular localization of the receptor in endocytic vesicles and vacuoles but also in lysosome-like bodies probably to a high extent reflects that this receptor also functions as Man-6-P receptor. Lysosome-like bodies with an electron dense appearance were heavily labeled with grains (Fig. 7c) after *in vitro* incubation on cryosections indicating that these are prelysosomes or primary lysosomes characterized by the presence of Man-6-P receptors [29]. The method presented in this study with direct labeling of IGF-II on ultrathin cryosections can thus be used for the identification of prelysosomes and late endosomes.

The localization of IGF-II/Man-6-P receptors in endocytic invaginations, endocytic vesicles, vacuoles, and in dense apical tubules, combined with the rapid internalization and intracellular processing of ^{125}I-IGF-II into lysosomes, raise the possibility of rapid IGF-II/Man-6-P receptor cycling between the cell surface and components of the vacuolar systems. Thus, the receptor probably follows the endocytic and recycling pathway for membrane constituents as previously described [30] and recently reviewed [31, 32].

In conclusion, the present study strongly suggests that IGF-II/Man-6-P receptors are located in the apical plasma membrane in endocytic invaginations in the proximal tubule but we cannot totally exclude the presence of small amounts in the basolateral membranes and/or glomeruli.

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